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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

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Application for Research Grant
(Use extra pages as needed)

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Date: 1/14/74

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3. Department(s) where research will be done or collaboration provided:

Division of Microbiology, Department of Laboratories.

4. Short title of study: The influence of acute and extended exposure to cigarette smoke on the immunological function and metabolic activity of alveolar macrophages as related to pulmonary defense against inhaled bacteria.

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims: The proposed project is a direct investigation of the influence of acute and extended exposure to puffed cigarette smoke on the immediate immunological function of alveolar macrophages in pulmonary defense; namely, the phagocytosis and destruction of inhaled bacteria. Methods are used that permit an evaluation of the effect of smoke-inhalation on the antibacterial capacity of alveolar macrophages under in vivo conditions of smoke-exposure and bacterial challenge. By means of paired and matched studies that utilize in vivo and in vitro quantitative methods of studying host defense mechanisms, it will be possible to study several immunological and biochemical parameters of alveolar macrophage function associated with antibacterial activity. Experiments are planned to permit the following evaluation of the effect of whole smoke and filtered smoke on the alveolar macrophage system and the reversibility of any adverse effects that may occur as a result of smoke inhalation:(a)phagocytosis and intracellular killing activity of alveolar macrophages under in vivo and in vitro conditions of bacterial challenge,(b)alveolar macrophage motility and cell adhesiveness as related to alveolar macrophage mobilization in response to inhaled bacteria and smoke(c) the antibacterial activity and phagocytosis promoting properties of alveolar lining material and pulmonary secretions,(d)secretory IgA levels present in the lungs of animals under basal conditions and after exposure to bacterial aerosols,(e)oxygen uptake, glucose metabolism, lipid composition and fatty acid composition of alveolar macrophages harvested from animals under basal conditions and after bacterial provocation and (f)ATPase, hydrolytic enzyme, catalase, peroxidase activities and hydrogen peroxide production before and after bacterial challenge. This fundamental approach supports the projects specific aim to assess the influence of smoke-exposure on the alveolar macrophage system and to provide a rational basis for explaining the smoke data obtained in this laboratory:namely, cigarette smoke caused an impairment of the antibacterial activity that was totally reversible, and not apparent in macrophages harvested from smoke-exposed animals and then challenged with bacteria in the absence of cigarette smoke.

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8. Brief statement of working hypothesis:

Background Material - Supporting Data - Working Hypothesis of Grant Proposal.

See pages 2A-1 to 2A-20

9. Details of experimental design and procedures (append extra pages as necessary)

See pages 2A-21 to 2A-33

8. Statement of Working Hypothesis and Supporting Data: (Cited references on pages 2A-17 to 2A-20)

There is a great deal of information which documents the capacity of the normal lower respiratory tract to clear bacteria and remain sterile. This is indeed remarkable since bacterial contamination by inhalation and aspiration are a common occurrence. Evidence for the antibacterial activity of bronchopulmonary tissue of human beings and experimental animals, respectively, resides in the observations that bronchial secretions of normal subjects are sterile (1-3) and that bacteria deposited in the lungs of animals are rendered nonviable within hours (4-6). However, other studies also show that the bronchial secretions of chronic bronchitic patients contain respiratory pathogens (3) and that various experimental conditions, of suggested clinical importance, impede the pulmonary defense of mice against inhaled bacteria (4, 6-9).

Quantitative studies of pulmonary resistance to infection have been made possible by the development of techniques for simulating the natural mode of respiratory infection, namely, the inhalation of droplet nuclei laden with bacteria. This laboratory has developed, standardized and used for the past 10 years an aerosol system which permits the production of bacterial aerosols of controlled concentration and particle size from which predictable numbers of bacteria can be implanted in the lungs and trachea of experimental animals. By this method rapid lung clearance of a coagulast positive strain of Staphylococcus aureus by the murine lung has been demonstrated. One hour after aerosol exposure, 45% of the bacteria deposited are cleared: after 2 hours, 70%: 3 hours 80%: 4 hours 88%, and after 6 hours, 95% of the staphylococci are cleared. This clearance curve is remarkably constant for staphylococcal depositions between 10,000 and 300,000. Tracheobronchial clearance of the same strain of S. aureus is also effective and very rapid. In one hour 72% of the bacteria deposited on tracheobronchial tissue were cleared; at 2 hours, 85% and at 4 hours, 95% of the bacteria were cleared. Similar lung clearance rates for the removal of the same strain of S. aureus has been reported by other investigators (6, 7). Published data from our laboratory indicates that hypoxia, barbiturates, cigarette smoke and alcohol introduced immediately after bacterial aerosol exposure cause a reduction in lung clearance (4, 8, 9). Other published reports from our laboratory demonstrate that pretreatment with cortisone or endotoxin interferes with normal clearance (4, 8, 9) and the intestinal flora of the mouse exerts a profound influence on pulmonary resistance to an airborne bacterial challenge. Reports by other investigators demonstrate that acute starvation (7), cold (6), bacterial species (6), pre-exposure to a viral infection (11), ozone (12), silicosis (13), renal failure (14) and acidosis (15) markedly affect the normal process of removal of bacterial deposits by pulmonary tissue. It is of interest that abnormalities in normal lung clearance correlate better with indices of physiological disfunction than with observable anatomical derangements. For example, experimental silicosis (13) caused minor reduction in lung clearance despite extensive anatomical derangement. In contrast, physiological circumstances such as acidosis (13) hypoxia (8), alcoholic intoxication (10) and barbiturates (8), cause inhibition of normal lung clearance without apparent damage to lung structure. Indeed the host factors which determine the outcome of the interaction between bacteria and lung tissue remain unclear. Reports from our laboratory clearly demonstrate the importance of genetic and environmental factors in pulmonary resistance to infection (8). We observed that two genetically related strains of mice differed solely in their capacity to withstand exposure to a single experimental condition, namely, cigarette smoke. It was also observed that the presence or absence of gram negative bacteria, specifically Escherichia coli predisposes mice to greater difficulty in handling inhaled bacteria, especially under the circumstance of smoke inhalation (8). In this regard, it has been demonstrated in our laboratory that circulating bacterial products such as E. coli endotoxin cause a marked interference with normal clearance of inhaled bacteria (8).

The mucociliary system and alveolar macrophages comprise the major defenses of the respiratory tract. The individual effectiveness of each component has been demonstrated several times (15, 16) and newer techniques have made it possible to study in detail their individual participation in the disposal of foreign material from the lung (17-20). Attention has been focused on the importance of alveolar macrophages by studies which demonstrate that clearance of bacteria that are deposited at alveolar levels,is not primarily achieved by mechanical removal of bacteria by the mucociliary apparatus but is accomplished by the in situ bactericidal action of the lung (19). In addition, published reports from this laboratory (21-22) indicate that alveolar macrophages are mobilized in response to an airborne bacterial challenge. The inhalation of staphylococci provoked a 2.5 fold increase in macrophage numbers. Within 15 minutes after bacterial deposition, the numbers dropped 35%; but the elevated levels observed immediately after aerosol exposure were restored at the end of 30 minutes and then maintained with minor variation for a 4 hour period. The observation that whole-body x-irradiation (23) and immunosuppressive agents (24) suppress normal bacterial clearance, adds additional support to existing evidence that alveolar macrophages play an important role in pulmonary defense against inhaled bacteria. By utilizing recent refined techniques for securing alveolar macrophages in almost pure harvest, it has been possible to study the antibacterial and biochemical properties of alveolar macrophages. Under these circumstances published reports demonstrate that the alveolar macrophage system responds to a variety of experimental conditions that are epidemiologically associated with the genesis of pulmonary disease (8, 25). In this regard, published reports from this laboratory indicate that alcohol interferes with the mobilization of alveolar macrophages in response to a bacterial challenge but does not adversely affect cell viability (22). In contrast, cigarette smoke provokes an increase in the number of macrophages present in the lungs and does not adversely effect cell viability or interfere with macrophage mobilization by bacterial provocation (26). Cigarette smoke under in vivo condition of acute exposure to puffed cigarette smoke was observed to cause an inhibition of antibacterial activity that was reversible within hours (27) and the alveolar macrophage may act as the major detoxification mechanism available to the normal lung during acute exposure to cigarette smoke. Other investigators have shown that various inhaled noxious agents affect the function and number of alveolar macrophages. These agents include nitrogen dioxide (28, 29), ozone (30) and lead sesquioxide (31). In this regard, ozone, nitrogen dioxide and auto smog adversely affect the cell numbers (28,30), phagocytic ability (28,30) viability (32), hydrolytic enzymes (33) and interferon production (29) of the alveolar macrophage. Recent studies demonstrated that the alveolar macrophage is a specialized cell with metabolic and enzymatic characteristics distinct from most other white blood cells with phagocytic properties (34, 35). Its basal oxygen consumption is four times that of polymorphonuclear leukocytes, yet it requires only 20% increase during active phagocytosis. Peritoneal macrophages require a 250% increment for similar activities. In addition, the alveolar macrophage depends upon oxidative energy metabolism for its phagocytic function, and it has 3 to 4 times the lysozyme, acid phosphatase, and beta-glucuronidase activity of peritoneal macrophages (35). Recent studies (36) indicate that phagocytosis by alveolar macrophages is accompanied by the production of carbon dioxide $^{14}C$ from glucose -1 $^{14}C$ which is at least 5 times that observed with glucose -6 $^{14}C$. The $^{14}CO_2$ production from both forms of labelled glucoses increases 4 times during active phagocytosis. The same laboratory (36) has presented data that suggests that alveolar macrophages exhibit peroxidative metabolisms and possesses 2 $H_2O_2$ utilizing pathways; namely, a nicotinamide adenine dinucleotide phosphate (NADP) linked cytoplasmic glutathione shuttle and a catalase dependent pathway. The significance of the $H_2O_2$ metabolism is uncertain. In the polymorphonuclear leukocyte, $H_2O_2$ constitutes an important component of an intracellular bactericidal system (36). Its role in the alveolar macrophage is less clear, since the alveolar macrophage lack white blood cell peroxidase, another important constituent of their bactericidal system. Several studies may be cited in which the metabolic

function of alveolar macrophages are deranged by experimental agents that also interfere with bacterial clearance and the antibacterial activity of alveolar macrophages; cadium (37) ions interfere with oxidative metabolism; and ozone depresses lysosomal hydrolase activity (38).

The role of the mucociliary apparatus as a major pulmonary defense mechanism has been recognized for years. It limits the number of inhaled particles that enter the lower respiratory tract and has the capacity to physically remove particles that are deposited at various levels of the lung endowed with ciliated cells. Inhaled bacteria that deposit on the ciliated surface of the trachea and bronchi are treated as particles and are subjected to the clearing action of the mucociliary apparatus. Certain environmental agents of clinical interest inhibit mucociliary function; alcohol (9), formic and acetic acid (39) and high concentrations of oxygen (40). Evidence for (41) and against (42) an adverse effect of smoke on particulate clearance of inhaled material may be cited. Viral infections (43), vitamin A deficiency (44) and chronic bronchitis (45) are specific diseases that affect the bronchial tract and also demonstrate an effect on the salient components of the mucociliary apparatus; namely, cilial motion, mucus production and particle clearance. The recent observation, that bronchial mucus contains antibodies and several substances that exert non-specific bacteriostatic and bacteriocidal action against gram positive and gram negative bacteria (46) suggests that mucus or its components may contribute directly to bacterial killing of inhaled bacteria per se or provide the local conditions necessary for efficient alveolar macrophage activity. To correlate with this recent studies suggest that alveolar lining material (47) and local immune systems (SIgA) (48) may play a significant role in pulmonary defense against inhaled bacteria.

The experimental approach and studies proposed in the current grant request emanate from observations made in this laboratory regarding the influence of acute and extended exposure to cigarette smoke on pulmonary defense against inhaled bacteria under in vivo conditions of smoke-exposure and bacterial challenge. For this reason, a detailed review of the data accrued to date is indicated.

Acute exposure to cigarette smoke altered the normal pattern of tracheal and lung clearance of bacteria.(see 8A supporting data, results section I(A) - I(C) pages 2A-10 ). The introduction of cigarette smoke immediately after exposure to staphylococcal aerosols resulted in a decrease in the number of viable bacteria present in the trachea and a concomitant increase in the numbers of bacteria retained in the lung. Under the acute conditions of these experiments, the effect of cigarette smoke on tracheal clearance was found to be reversible. Alveolar macrophage studies have provided the following pertinent information. Smoke inhalation provoked a dose related increase in basal macrophage numbers and did not adversely affect cell viability. In addition, exposure to cigarette smoke after staphylococcal aerosol challenge did not interfere with the mobilization of macrophages in response to a bacterial challenge. These findings demonstrate that one of the immediate effects of smoke inhalation on the cellular defense mechanisms of the respiratory tract is a direct stimulation of the alveolar defense system without an adverse effect on cell viability. To correlate with this, smoke-exposure did not provoke the migration or mobilization of other phagocytic cells such as polymorphonuclear leukocytes to pulmonary sites. The data further suggests that the presence of increased macrophages may play a central role in protecting the lung against smoke toxicity. To postulate that these cells play a protective role is supported by studies presented as supporting data in this report which demonstrate the following: (1) increased numbers of macrophages reduce or limit the adverse effect of cigarette smoke on the antibacterial activity of a macrophage population in vitro and (2) alveolar macrophages are capable of inactivating toxic components present in a smoke solution that impair the

the antibacterial activity of normal alveolar macrophages in vitro.

The data obtained from a direct investigation of the phagocytic and bactericidal properties of alveolar macrophages under in vivo conditions of smoke-exposure has provided additional pertinent information. (see 8A Supporting Data, Results Section II(A) - II(C) pgs.2A-11 to 2A-12)As demonstrated by the pulmonary lavage studies, exposure to cigarette smoke caused an accumulation of "toxic substances" that inhibit the antibacterial activity of alveolar macrophages. However, smoke inhalation did not permanently impair the antibacterial activity of the alveolar defense system. Specifically, smoke-exposure caused an impairment of the phagocytic and bactericidal powers of macrophage harvests that was readily reversible within hours. The fact that macrophage function was restored under in vitro conditions of recovery; namely by multiple washing and incubation of macrophage in a smoke-free environment demonstrates the capacity of the alveolar macrophage system to cope with cigarette smoke and the importance of in vivo processes which effectively remove inhaled products of cigarette smoke that influence macrophage function either directly or indirectly by altering the immediate environment in which macrophages perform their phagocytic function. To correlate with this, the adverse effect of smoke inhalation was reversed under in vivo conditions of recovery, namely by housing smoke-exposed animals in a smoke-free room prior to obtaining lung harvests. Collectively, these observations suggest that the respiratory tract responds to the presence of cigarette smoke, as well as other foreign materials in a similar manner; namely, pulmonary defense mechanisms are activated and respond by removing and/or inactivating smoke products deposited in the lung. It appears reasonable to assume that the efficacy of this response is facilitated by the in vivo mobilization of increased macrophage numbers in relation to the inhalation of cigarette smoke. Such an interpretation of the data would be in keeping with experimental evidence previously cited in this report which demonstrates that alveolar macrophages may play a key role in protecting the lung against the toxic effects of cigarette smoke.

Studies performed under in vitro conditions of smoke-exposure have served to provide: (1) the laboratory techniques necessary for more meaningful studies of the effect of cigarette smoke on the living and functioning lung and (2) a better understanding of the conditions of bacterial challenge and smoke-exposure that influence the antibacterial properties of alveolar macrophages. As demonstrated by studies presented in this report (see 8A Supporting Data, Results Section III (A) - III (F) pgs.2A-12 to 2A-15)the antibacterial capacity of a macrophage population in the absence and presence of cigarette smoke was enhanced by the presence of increased numbers of alveolar macrophage. Specifically, the presence of increased numbers of macrophages reduced or limited the immediate adverse effect of cigarette smoke on alveolar macrophage function. In addition, macrophage cultures inactivated toxic components of a smoke solution that impair the antibacterial activity of alveolar macrophages in vitro. These findings support published reports which have focused attention on the importance of alveolar macrophage mobilization as a pulmonary defense mechanism against inhaled infectious and toxic agents. Results of other studies presented in this report indicate that the local conditions necessary for effective antibacterial activity by normal and smoke-exposed macrophages differ. In this regard, the antibacterial activity of macrophages during smoke-exposure was significantly influenced by the concentration of serum in the culture medium. This effect could not be accounted for by the presence or absence of an added carbon source (glucose) in the culture medium or the increased acidity of the culture medium induced by cigarette smoke. The fact that exposure to cigarette smoke interfered with the antibacterial activity of alveolar macrophages only at serum concentrations of 2.5% or less indicates that serum factors may protect against smoke toxicity. This may be related to specific immunological components of serum. In addition, it has also been observed that the protective action of serum is reduced if cigarette smoke is introduced prior to

bacterial challenge. Specifically, prior exposure of macrophage cultures to cigarette smoke reduced the amount of cigarette smoke necessary to suppress antibacterial activity. This difference may be a reflection of the long term effects of cigarette smoke on a static macrophage population and probably relates to several factors including the effect of extended exposure to cigarette smoke on: (1) serum factors which promote and sustain effective macrophage function and (2) the physiological state of macrophages at the time of bacterial challenge. Macrophages obviously respond to the presence of cigarette smoke by removing and/or detoxifying smoke products in their environment. The fact that the adverse effects of in vitro smoke-exposure was partially reversed by multiple washing of macrophage cultures demonstrates the beneficial results derived from reducing the amount of smoke in contact with the alveolar cells and the importance of the cleansing action of increased numbers of macrophages (mobilization) and mucociliary activity in the live and functioning lung. Data has also been presented which clearly demonstrates that exposure to whole smoke drawn through a glass fiber filter reduced the adverse effect of in vitro smoke-exposure on the antibacterial activity of macrophages. Specifically, filtered whole smoke did not interfere with phagocytic activity but continued to impair bacterial killing. These observations suggest that the particulate and gas phases of whole smoke may have separate effects on macrophage function.

Marked differences in the effect of in vivo and in vitro smoke-exposure on macrophage function have also been noted. In vitro conditions of smoke-exposure produced an impairment of antibacterial activity that was not totally reversed by multiple washing and incubation of macrophages in smoke-free tissue culture flasks. The differences in the effect of in vivo and in vitro smoke-exposure on macrophage function may be attributable to several factors including: (1) differences in the amount of smoke that macrophages were exposed to under both experimental conditions and (2) the static and unphysiological conditions that prevail under in vitro conditions of smoke-exposure. The latter interpretation of the data would be in keeping with the mobilization and detoxification data reported herein.

Pulmonary studies of the influence of extended exposure to puffed cigarette smoke on pulmonary defense against inhaled bacteria under in vivo conditions of smoke-exposure and bacterial challenge have also been completed (see 8A Supporting Data, Results Section IV(A)-IV(D) pgs.2A-15 to 2A-16). Exposure to cigarette smoke for one hr daily for 3, 5, 10 and 15 days prior to bacterial aerosol challenge altered pattern of normal clearance. Specifically, smoke inhalation reduced the initial clearance rate of inhaled bacteria but normal clearance rates were restored with minor variation within hours after the termination of smoke-exposure. Under the conditions of these experiments, smoke inhalation provoked a large increase in basal macrophage numbers and did not adversely affect cell viability. In addition, cigarette smoke did not mitigate the mobilization of alveolar macrophages in response to bacterial provocation or provoke the presence of polymorphonuclear leukocytes in lung harvests. The data obtained from studies of the phagocytic and bactericidal properties of alveolar macrophages under in vivo conditions of extended exposure to cigarette smoke has provided the following information. The antibacterial activity of alveolar macrophages harvested from smoke exposed animals washed several times prior to bacterial challenge was unimpaired. A full report of these observations, as well as others pertaining to the influence of extended exposure to cigarette smoke on pulmonary defense will be submitted at the end of the current year of grant support.

The proposed grant request is a direct extension of the work completed in this laboratory concerning the influence of acute and extended exposure to cigarette smoke on pulmonary defense mechanisms. This project is designed on the basis of established evidence that alveolar macrophages play a dominant role in disposing of inhaled bacteria (18,19,21) and takes into consideration current information relating to the

presence in the bronchopulmonary tree of secreted fluids, both immunological and and non-immunological in character (49) that contribute to pulmonary defense and may act as the mediators of effective alveolar macrophage function. In addition, the grant proposal takes into account several biochemical parameters of alveolar macrophage function that are associated with the effective antibacterial activity of phagocytes. To correlate with this, evidence may be cited which demonstrates an apparent contradiction; namely, the effective antibacterial powers of alveolar macrophages in the intact and functioning lung(4-9) and the diminished bactericidal capacity (50,51) under in vitro conditions of study. The experimental design also acknowledges that meaningful animal studies must be performed under in vivo conditions of smoke-exposure and utilizes a smoke generating and exposure chamber that produces puffed cigarette smoke of predictable and controlled concentration. In this regard, the results obtained in this laboratory under in vivo conditions of smoke-exposure (See Supporting Data, Results Section,pgs.2A-10 to 2A-16) demonstrated that smoke-exposure produced an impairment of the antibacterial activity of alveolar macrophages that was readily reversible and not apparent in the absence of cigarette smoke or smoke products. These findings are in good agreement with the data obtained from studies (52) with alveolar macrophages harvested from human smokers and are at variance with the results (53) obtained with rabbit alveolar macrophages exposed to cigarette smoke under in vitro conditions of smoke-exposure.

Experiments are detailed which permit a direct investigation of the influence of acute and extended exposure to cigarette smoke on the immediate immunological functions of alveolar macrophages in pulmonary defense; namely, the phagocytosis and intracellular destruction of inhaled bacteria. By utilizing in vivo and in vitro quantitative methods of studying phagocyte response to bacterial provocation, studies are planned to investigate several parameters of alveolar macrophage function, associate with its central role in pulmonary defense against inhaled bacteria in the normal situation and during the inhalation of cigarette smoke. To this end, studies are planned to investigate the effect of smoke inhalation on the phagocytic and intracellular killing activity under in vivo and in vitro conditions of bacterial challenge. Separate studies are detailed to assess the influence of cigarette smoke on macrophage mobility and adhesiveness because they represent critical aspects in the in vivo mobilization of macrophages in response to an airborne bacterial challenge and cigarette smoke. Additional studies are proposed to evaluate the influence of alveolar lining material, Secretory IgA and pulmonary secretions on antibacterial activity of alveolar macrophages harvested from control and smoke-exposed animals. The information is needed to delineate the relative role of alveolar macrophages and secreted pulmonary fluids in pulmonary defense and to assess the relative susceptibility of each component to smoke inhalation. In addition, metabolic studies will be performed to assess the effect of cigarette smoke on ATPase activity, respiration, glucose metabolism, lactic acid production, catalase activity, peroxidase activity, hydrogen peroxide production, lipid composition, fatty acid composition, hydrolytic enzyme activity of alveolar macrophages which underlie or are concomitant with normal antibacterial activity. This information is desirable, since respiration, glucose metabolism, ATPase activity and lipid metabolism represent important links in the successful mobilization and subsequent antibacterial activity of alveolar macrophages. Indeed impaired lipid metabolism may compromise membrane phenomena associated with phagocytosis and derangements of peroxidative metabolism and/or the intracellular distribution and activity of hydrolytic enzymes may profoundly affect the bactericidal capacity of lung phagocytes. This fundamental approach supports the project's specific design to investigate: (a) the influence of acute and extended exposure to cigarette smoke on the immediate immunological function of alveolar macrophages in pulmonary defense against inhaled bacteria and (b) provide a better understanding of the relative role of alveolar macrophages in the cellular response of the respiratory tract, in the normal situation and during the inhalation of cigarette smoke.

8 (A) Supporting Data

## Materials and Methods

I. Conditions of Bacterial Clearance Studies: Since bacterial clearance studies comprise a major part of the proposed research project, a review of the method of aerosol formation, exposure and quantitative aspects of bacterial clearance is indicated. Aerosols are generated from a buffered suspension (pH 7.3) of staphylococci contained in 8 glass nebulizers. The initial spray from the nebulizers is directed into mixing chambers through which a secondary airflow of 100 cubic feet/min. is drawn. The large volume of secondary air serves to mix, dilute and dry the initial bacterial aerosol; direct it past an interposed baffle for removal of large droplets; and then carry it through a large plexiglass exposure chamber that can accommodate up to 200 mice. The particle size distribution of the bacterial aerosol is monitored with an Andersen Sampler. White male Swiss Webster mice are divided into groups of 8 to 10 animals and exposed to Staphylococcal aerosols for 30 min. Immediately after exposure (0 time), one group of animals is sacrificed to determine the numbers of viable bacteria deposited in the trachea and lungs, respectively. The trachea and lungs are removed as separate blocks and individually ground in glass homogenizers. The remaining groups of challenged mice are killed and processed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after aerosol exposure. Bacterial counts are obtained from nutrient agar pour plates of lung and tracheal tissue homogenates. By this method paired studies of lung and tracheal clearance of bacterial deposits are possible. By subtracting the mean number of culturable bacteria retained in the lung at each interval during the post aerosol exposure period from the number originally deposited (0 time) the mean number of staphylococci cleared by the lung may be derived. From these data lung clearance rates are calculated by expressing the number retained in the lung as a percentage of the deposition number (0 time) and subtracting this value from 100%. Similarly, from the numbers of bacteria present in the trachea at 0 time and at various intervals after aerosol exposure, the number and percent bacteria cleared by the trachea may be obtained.

II. Conditions of In Vivo Smoke-Exposure: It is apparent that meaningful studies of the influence of cigarette smoke on pulmonary resistance against inhaled bacteria require strict experimental circumstances. The concentration and composition of the smoke must be controlled and clearly defined. Variations in smoke generation are invariably accompanied by changes in the amount of tar and may influence the concentration and/or composition of the volatile components of the gas phase. In addition, studies under in vivo and in vitro conditions of smoke exposure demonstrate the importance of a dose-response relationship in the interpretation of the biological effects of cigarette smoke on the major defense mechanisms of the lung; namely, mucociliary function and alveolar macrophage activity.

The smoke exposure system is schematically presented in Figure I. Puffed cigarette smoke generated from non-filtered, 70 mm cigarettes is drawn into a stainless steel mixing chamber and through a smoke exposure chamber by a secondary airflow of room air. Animals in the control chamber are exposed to puffed air drawn through an unlighted cigarette directed through a stainless steel mixing chamber by a secondary air flow. The smoke exposure and control chambers are made of transparent plexiglass which permit observations of animals during experiments. Trap doors located on the top of each chamber facilitate the introduction and removal of a 4-tiered stainless steel rack that accommodates 40 mice housed in individual rubber mesh containers. The downstrem ends of the smoke exposure and control chambers are attached to separate stainless steel evacuation chambers that are connected by a common cylinder to the negative pressure side of an air blower. Sampling ports located in the evacuation chambers permit the introduction of sampling devices into the exposure chambers to obtain data relative to the particulate and gas phases of cigarette smoke, temperature and

relative humidity. The effluent from both the smoke exposure and control chambers is ultimately eliminated through a pipe inserted into an air duct that is vented outside the building. As shown in Figures 1A and 1B, a continuous stream of puffed cigarette smoke is produced by an automatic smoke machine* designed to sequentially puff 30 cigarettes and adjusted to deliver a 35 ml puff of 2 seconds duration from each cigarette once every minute. The smoke generating apparatus consists of an index disc and wheel assembly programmed to position and maintain each lighted cigarette for 2 seconds at the intake port of the smoke aspirator. An airflow of 1.05 liters per minute through the intake port was established by introducing compressed air at a rate of 14.0 liters per minute into the air inlet of the smoke aspirator. Air flow into the air inlet of the smoke aspirator is measured with a calibrated rotometer and controlled by a needle point valve positioned between the air compressor and rotometer. The smoke exhaust of the aspirator is placed inside the open end of a stainless steel mixing chamber in which the cigarette smoke is further diluted and directed across the smoke exposure chamber by a flow of room air through the apparatus. This secondary air flow is established by the air blower at the downstream end of this apparatus. A rotometer and needle point valve are used to control the volume and rate of air flow.

The aspirator was calibrated by taking simultaneous measurements of the air flow at the air inlet and smoke exhaust orifices with a rotometer, pneumotachograph, electronic amplifiers and recorders. From these data, the gas volume drawn through a cigarette positioned at the smoke intake port was derived. The relationship between the volume of air introduced into the air inlet and drawn through a lighted cigarette is plotted in Figure II.

Separate studies were undertaken to obtain accurate determination of the volume and flow rate of room air through the apparatus. For this purpose an oriffice meter and manometer were introduced into the evacuation chambers of the control and smoke chambers as indicated in Figure III. Simultaneous measurements taken with a manometer and rotometer provided the data plotted in Figure IV. These results indicate that a secondary air flow of 30 to 100 liters per minute may be maintained in the smoke exposure chamber.

The data obtained from the above experiments has provided the information and techniques necessary to: (a) define the conditions of smoke exposure during studies of the influence of cigarette smoke on pulmonary resistance to infection. (b) standardize the conditions of smoke exposure from study to study. (c) assess the effect of different concentrations of cigarette smoke on alveolar macrophage and mucociliary function, (d) plan and implement a protocol to monitor the particulate and gas phases of cigarette smoke during animal studies and (e) assess the relative importance of the particulate and gas phases of cigarette smoke on host resistance to pulmonary infection.

III. Conditions of In Vitro Smoke-Exposure: A commercial brand of non-filtered cigarette was attached to a 30 ml. syringe by a rubber tube and smoke was produced by withdrawing the barrel of the syringe at a rate of approximately 18 ml. per second. Six successive "puffs" of cigarette smoke were introduced into the syringe and emptied by removing a rubber tube containing the lighted cigarette. The smoke from the seventh puff was introduced into the tissue culture flask with a sterile hypodermic needle. The protocol of each study included control flasks containing only bacteria and alveolar macrophages challenged with bacteria respectively. Under these conditions, both categories of tissue culture preparations were exposed for 1.5 hours to whole cigarette smoke or whole smoke drawn through a glass fiber filter**.

*Progressive Engineering Co., Richmond, Va.
** Cambridge glass fiber filter, Phipps and Bud, Richmond, Va.

IV. In Vitro Phagocytosis System: Alveolar macrophages were harvested from the lungs of rabbits weighing 1.0 to 2.0 kg by pulmonary lavage. The animals were sacrificed by injecting air into the external marginal vein. The trachea and lungs were exposed and macrophages harvested by washing out the intact lungs two times with 17 ml of the suspending medium used in the in vitro phagocytosis system (described below). The harvested cellular lung contents were recovered by centrifugation at 2000 rpm for 20 min. Total cell counts were performed in a bright line hemacytometer, and differential counts made on Wright stain smears.

The harvested alveolar macrophages were suspended in Hank's solution, diluted to a final cell count of 2.0 to 4.0 x $10^6$ cells and transferred into 30 ml plastic tissue culture flasks. The total fluid volume in each flask was 3.0 ml containing 2.0 to 4.0 x $10^6$ macrophages, 2.0 to 4.0 x $10^6$ Staphylococcus aureus and normal rabbit serum at various concentrations in accordance with the protocol of each study. The pH of control and macrophage containing flasks was determined with a Beckman zeromatic pH meter immediately after inoculation and at the end of each incubation period.

Quantitative studies of the phagocytic and intracellular killing activity of alveolar macrophages require the enumeration of the total number of viable bacteria present in the extracellular fraction and macrophage fraction of each preparation. In addition, the stability of the bacteria in the suspending medium as well as multiplication of extracellular bacteria must be monitored. For this purpose, control flasks devoid of macrophages but containing suspending medium were included in the protocol of each experiment. In every study, several flasks containing macrophages and control flasks were inoculated with bacteria. Immediately after bacterial challenge (0 time) and at various intervals after incubation at 37°C, replicate flasks were removed from the incubator and processed. The extracellular fraction was isolated by removing the supernatant from the macrophage cultures with a pipette and washing the macrophage monolayer 3 times with 2.5 ml of suspending medium. To recover the macrophage fraction, cell monolayers were washed 3 times with 2.5 ml of sterile distilled water to lyse the macrophages adhering to the surface of the tissue culture flask. To insure complete separation of the cells from the flask, 1 ml of glass beads was added after the addition of distilled water and shaken on a Vortex mixer for 1 min. The completeness of cell separation was confirmed by microscopic examination of the tissue culture preparation. The contents of the control flasks were removed with a pipette and the flasks washed 3 times with suspending medium. The extracellular fraction, macrophage preparation and contents of control flasks were placed in an ice water bath and bacterial counts were obtained from nutrient agar pour plate incubated at 37°C for 24 hrs. Bacterial counts were taken from separate pairs of control and macrophage containing flasks immediately after introduction of S. aureus (0 hr.) and at various intervals after incubation at 37°C. From the number of viable S. aureus present in the control flask (A) extracellular fraction (B) and macrophage fraction (C) at each interval after bacterial challenge, the % bacteria phagocytized, % phagocytized bacteria surviving within macrophages and % bacteria cleared by macrophages were determined as follows:

(1) $\%\ \text{phagocytized} = \frac{A - B}{A} \times 100$ (2) $\%\ \text{intracellular survival} = \frac{C}{A - B} \times 100$

(3) $\%\ \text{killed} = \frac{B + C \text{ at each interval after bacterial challenge}}{B + C \text{ at 0 hr.}} \times 100 - 100\%$

Separate studies were performed in which inactivation of intracellular bacteria was assessed independent of phagocytosis. For this purpose several macrophage cultures were challenged with S. aureus as described above for 30 min. At this time, the supernatant was discarded and the surface of each macrophage cultures washed with Hank's solution to remove all extracellular bacteria and thus terminate the phagocytosis of additional bacteria. Bacterial counts were obtained in triplicate immediately after termination of phagocytosis and at various hourly intervals.

## Results

I. The Influence of Acute Exposure to Puffed Cigarette Smoke on Pulmonary Clearance of Inhaled Staphylococcus Aureus Under In Vivo Conditions of Bacterial Challenge and Smoke-Exposure.

(A) The Effect of Acute Exposure to Cigarette Smoke on Tracheal and Lung Clearance: Large numbers of mice were exposed to aerosols of S. aureus for 30 minutes. Immediately after aerosol exposure one group of mice was sacrificed to determine the number of bacteria deposited in the trachea and lungs, respectively. The remainder of the bacteria-challenged mice were divided into smoke and control groups and exposed to cigarette smoke and a secondary air flow of room air, respectively. As shown in Table I, within 15 minutes after exposure to cigarette smoke, there was a 2.5 fold decrease in the numbers of staphylocccci culturable from the trachea, and this decrease in viable bacteria was observed with minor variation throughout the remainder of the 4 hour post aerosol exposure period. This effect was accompanied by an increase in the numbers of bacteria retained in the lung. At the end of 30 minutes, the smoke exposed mice retained 1.4 times as many bacteria as controls. Bacterial retention was further increased 2.1 to 2.5 times control numbers by the inhalation of cigarette smoke for 1 and 4 hours, respectively. As shown in Table II the effect of cigarette smoke on tracheal clearance was reversible. Within 3 hours after the termination of smoke exposure, bacterial counts obtained from the trachea of control and smoke exposed mice were not significantly different.

Conclusions: Cigarette smoke altered the normal pattern of tracheal and lung clearance. Smoke inhalation resulted in a decrease in the number of bacteria present in the trachea and a concomitant increase in the number of viable bacteria retained in the lungs. The effect of cigarette smoke on tracheal clearance was reversible.

(B) The Effect of Cigarette Smoke on Basal Macrophage Yields: The effect of cigarette smoke on the numbers of alveolar macrophages harvested from the lungs of mice was studied by comparing the macrophage yields obtained from untreated mice (Basal Group), mice exposed to a secondary air flow (Control Group) and animals challenged with puffed cigarette smoke diluted by a secondary air flow. The data presented in Table III demonstrates that cigarette smoke provokes a reproducible increase in macrophage numbers. The latter effect was found to be dose related. In addition, exposure to cigarette smoke for 4 hours did not adversely affect macrophage viability (Table IV).

Conclusions: Smoke inhalation provokes a dose related increase in basal macrophage numbers and does not adversely affect cell viability.

(C) The Effect of Cigarette Smoke on the Mobilization of Alveolar Macrophages in Response to a Bacterial Challenge: For this purpose basal macrophage yields were obtained from untreated mice and the remainder were exposed to an aerosol of S. aureus for 30 minutes. Immediately after aerosol exposure one group of mice was sacrificed to determine the numbers of bacteria deposited in the lung and the remainder of the challenged mice were divided into Control and Smoke groups. As indicated in Tables V and VI, the macrophage yields obtained from Control and Smoke exposed mice were the same over an entire 4 hour post aerosol exposure period.

Conclusions: Smoke inhalation did not interfere with the mobilization of alveolar macrophages in response to a bacterial challenge.

II. The Influence of Acute Exposure to Puffed Cigarette Smoke on the Antibacterial* Activity of Alveolar Macrophages: In Vivo Smoke-Exposure and In Vitro Bacterial Challenge:

(A) The Antibacterial Activity of Alveolar Macrophages Incubated in the Pulmonary Lavage Solution Obtained From the Lungs of Control and Smoke-Exposed Rabbits: For this purpose, the lungs of smoke-exposed and control rabbits were washed with Hank's solution containing 0.1% glucose and 2.5% normal rabbit serum. The recovered lung lavage was then separated by centrifugation into an acellular fraction (pulmonary lavage solution) and a cellular fraction containing macrophages. In each study, the antibacterial activity of control macrophages was evaluated after incubation for 1.5 hours in pulmonary lavage solution obtained from smoke-exposed and control animals.

As shown in Table VII, incubation of control macrophages in control pulmonary lavage solution resulted in effective antibacterial activity. At the end of 1.5 hours, the macrophage cultures phagocytized 55% and killed 40% of the staphylococci. In contrast, control macrophages incubated in pulmonary lavage solution obtained from smoke-exposed animals showed a 27% decrease in the percent bacteria phagocytized and a 25% decrease in the percent bacteria killed.

Conclusions: The impairment of the antibacterial activity of alveolar macrophages by cigarette smoke inhalation may be directly related to the presence or accumulation of "smoke products" in the lung. "Smoke products" may exert their adverse effect on the alveolar macrophage defense system directly by compromising the antibacterial capacity of existing macrophages or indirectly by altering the immediate environment in which macrophages perform their phagocytic function.

(B) The Effect of Cigarette Smoke on the Antibacterial Activity of Alveolar Macrophages: In Vivo Smoke-Exposure and In Vitro Bacterial Challenge. In order to compare the antibacterial activity of smoke-exposure and control rabbits, experimental and control animals were exposed to puffed cigarette smoke and the secondary air flow, respectively, for 1.5 hours. At this time, alveolar macrophages harvested on both categories of animals were placed in tissue culture flasks and immediately challenged with S. aureus. Other studies were performed to determine if changes in the antibacterial activity of macrophages harvested from smoke-exposed animals were reversible. The reversibility studies were performed under two different experimental recovery conditions. In one series of studies, macrophage harvests from smoke-exposed animals were incubated in tissue culture flasks for 1.5 hours (in vitro recovery) prior to bacterial challenge. In the second study series, in vivo circumstances of recovery were established. Rabbits were first exposed to cigarette smoke for 1.5 hours and then placed in a smoke-free room for another 1.5 hours. At this time, the macrophages were harvested and challenged with staphylococci. Appropriate controls were included in every study. As shown in Table VIII, the antibacterial activity of alveolar macrophages harvested from animals exposed to whole cigarette smoke for 1.0 and 1.5 hours, respectively, was impaired. The data presented in Table IX clearly demonstrates that the adverse effect of cigarette smoke on the antibacterial activity of alveolar macrophage was reversed by incubation in a smoke-free tissue culture flask for 1.5 hours prior to bacterial challenge (in vitro conditions of recovery). Similarly, as shown in Table X, the smoke effect was reversible under in vivo conditions of recovery; specifically, the alveolar macrophage harvested from smoke-exposed animals housed in a smoke-free room for 1.5 hours prior to macrophage harvest phagocytized and destroyed staphylococci as effectively as macrophages from control animals.

Conclusions: Exposure of alveolar macrophages to whole cigarette smoke under in vivo conditions of smoke-exposure caused an impairment of the antibacterial activity of alveolar macrophages that was reversible within hours.

* % changes in antibacterial activity of smoke-exposed macrophages derived as follows: % phagocytosis by control minus % phagocytosis by smoke-exposed; % killed by controls minus % killed by smoke-exposed macrophages.

(C) The Effect of Multiple Washings of Alveolar Macrophages Harvested from Smoke-Exposed Animals on Cigarette Smoke Induced Changes in Antibacterial Activity: Studies of the reversibility of the adverse effect of cigarette smoke on macrophage function were performed under in vitro condition of recovery. Macrophages harvested from smoke-exposed and control animals were washed three times with Hanks' solution and then challenged with S. aureus. In addition, each study also included macrophage harvests from both categories of animals that were not washed prior to bacterial challenge. As shown in Table XI, compared to unwashed controls, the percent bacteria phagocytized by unwashed macrophages harvested from smoke-exposed animals was decreased 22%, the percent intracellular survival of phagocytized bacteria was increased 26%, and the percent bacteria killed decreased 25%. In contrast, the alveolar macrophages from smoke-exposed animals washed prior to bacterial challenge showed the same phagocytic and bactericidal powers as macrophages harvested from control animals.

Conclusions: Multiple washing of alveolar macrophage harvested from smoke-exposed animals completely reversed the adverse effect of smoke inhalation on macrophage function.

III. The Effect of Cigarette Smoke on the Antibacterial Activity* of Alveolar Macrophages: In Vitro Smoke-Exposure and In Vitro Bacterial Challenge: For this purpose alveolar macrophages harvested from untreated rabbits were placed in tissue culture flasks containing Hanks' solution supplemented with 0.1% glucose and 2.5% normal rabbit serum. In one series of studies, 8 ml. of cigarette smoke from a commercial brand of non-filtered cigarette, were introduced and maintained for 1.5 hours immediately after bacterial challenge. Under these conditions, studies were performed to determine the influence of alveolar macrophage numbers on the bactericidal capacity of an in vitro antimicrobial system during smoke-exposure. In a second series of studies, macrophage cultures were first exposed to cigarette smoke for 1.5 hours, and then challenged with bacteria. Cigarette smoke was introduced as an experimental condition by subjecting macrophages to either 8 ml. of whole smoke or Hanks' solution exposed to 2 ml. of whole smoke for 1.5 hours prior to use as a culture medium. Under these circumstances of smoke-exposure, studies were performed to determine if changes in the antibacterial activity of smoke-exposed macrophages were reversible. In addition, "detoxification" studies were carried out to determine the ability of alveolar macrophages to eliminate constituents of smoked Hanks' solution that depress antibacterial activity.

(A) The Influence of Macrophage Numbers on the In Vitro Antibacterial Activity of Alveolar Macrophages During Smoke-Exposure: Studies were performed in which macrophage-bacterium ratios (M/B) of 2:1, 3:1 and 4:1 were established by challenging $4 \times 10^6$, $6 \times 10^6$, $8 \times 10^6$ macrophages, respectively, with $2 \times 10^5$ staphylococci.

The data presented in Table XII indicate the following: (1) at each macrophage-bacterium (M/B) ratio studied, exposure to 8 ml. of cigarette smoke interfered with the phagocytosis and intracellular destruction of bacteria and (2) as the ratio of macrophages to bacterium (M/B) is increased, there is a significant decrease in the adverse effect of 8 ml. of cigarette smoke on the antimicrobial properties of alveolar macrophages. As shown in Table XII, at a M/B ratio of 2:1, the percent bacteria killed by smoke-exposed macrophages was decreased 27% compared to corresponding controls. In contrast, at M/B ratios of 3:1 and 4:1, the percent staphylococci killed by smoke-exposed macrophages was decreased 20% and 12%, respectively.

Conclusions: The presence of increased numbers of macrophages reduces or limits the adverse effect of cigarette smoke on alveolar macrophage function in an in vitro antimicrobial system.

* % changes in antibacterial activity of smoke-exposed macrophages derived as follows: % phagocytosis by control minus % phagocytosis by smoke-exposed; % killed by controls minus % killed by smoke-exposed macrophages.

(B) The Action of Alveolar Macrophages on the "Toxic" Components of Smoked Hanks' Solution: A smoke solution was prepared by introducing 2 ml. of whole smoke into 30 ml. tissue culture flasks containing 3 ml. of Hanks ' solution supplemented with 0.1% glucose and 2.5% normal rabbit serum. Alveolar macrophage harvests obtained from several rabbits were pooled and used in experiments specifically designed to provide the following information: (1) the effect of a smoke solution on the antibacterial activity of alveolar macrophages, (2) the reversibility of changes in macrophage function induced by the smoke solution and (3) the effect of alveolar macrophages on the constituents of a smoke solution that may influence antibacterial activity. This was accomplished by assessing the antibacterial activity of alveolar macrophages under the following conditions of bacterial challenge: (A) Experimental Conditions I - In order to investigate the immediate toxic effect of the smoke solution on antibacterial activity, macrophage cultures were exposed to smoked Hanks' solution and S. aureus introduced simultaneously and maintained for 1.5 hours, (B) Experimental Conditions II - Reversibility studies were performed in which macrophage cultures were first exposed to smoked Hanks' solution for 1.5 hours. At this time, the smoke solution was removed by pipette and set aside for future use. The macrophage cultures were then provided with fresh unsmoked Hanks' solution and challenged with staphylococci for 1.5 hours and (C) Experimental Condition III - To study the influence of alveolar macrophage activity on the components of a smoke solution that affect macrophage function, macrophage cultures were challenged with S. aureus during incubation in the smoke solution previously set aside after recovery from the tissue culture flasks described in Experimental Condition II. The bacteria and recovered smoke solution were introduced simultaneously and maintained for 1.5 hours.

The data presented in Table XIII indicates that smoked Hanks' solution caused an irreversible impairment of the antibacterial activity of alveolar macrophages. As shown in Table XIII (Experimental Condition I) compared to controls, macrophage cultures exposed to smoked Hanks' solution and S. aureus simultaneously, showed a 16% decrease in the percent bacteria phagocytized and an 18% decrease in the percent bacteria killed. Comparable results were obtained with macrophage preparations exposed to smoked Hanks' solution for 1.5 hours and then challenged with bacteria in unsmoked Hanks' solution (Experimental Condition II). The data also demonstrates that smoked Hanks' solution previously used as a suspending medium for alveolar macrophages loses its ability to impair antibacterial activity. As shown in Table XIII (Experimental Condition III) macrophages challenged with bacteria in smoked Hanks' solution previously subjected to alveolar macrophage activity for 1.5 hours, phagocytized and destroyed staphylococci as effectively as control macrophages.

Conclusions: Smoked Hanks' solution caused a marked impairment of the antibacterial capacity of alveolar macrophages. This toxic effect is eliminated by subjecting smoked Hanks' solution to alveolar macrophage activity. It appears reasonable to assume that alveolar macrophages are capable of reducing or eliminating the water soluble constituents of the gas and/or particulate phase of whole cigarette smoke responsible for depressed antibacterial activity, in vitro. Whether this effect represents physical adsorption of smoke products on the surface of macrophages or phagocytic removal and ultimate detoxification of smoke products by alveolar macrophages remains unclear.

(C) The Influence of Serum Factors on Macrophage Function During Smoke-Exposure: For this purpose, alveolar macrophages harvested from untreated rabbits were incubated in tissue culture flasks containing Hanks' solution with and without 0.1% glucose containing various concentrations of normal rabbit serum. Cigarette smoke from a commercial brand of non-filtered cigarettes was introduced immediately after bacterial challenge (S. aureus). As shown in Table XIV, the efficacy of macrophage function during smoke-exposure is influenced by the concentration of serum in the suspending medium.

In Hanks' solution (0.1% glucose) containing 5.0% serum, the antibacterial activity of alveolar macrophages was not adversely affected by introducing 1, 2, 4 and 8 ml. of whole cigarette smoke immediately after bacterial challenge. However, at a serum concentration of 2.5%, 8 ml. of smoke suppressed antibacterial activity. Under culture conditions in which the serum concentration was reduced to 1.0% and 0.5% the antibacterial activity of macrophages was reduced by the presence of 4 and 8 ml. of cigarette smoke. Studies in which the concentration of serum was varied have also been performed with Hanks' solution without glucose (Progress Report No. 1). Under these conditions, serum exerted the same influence on macrophage function during smoke exposure as noted above.

Conclusions: The efficacy of macrophage function during smoke-exposure was not affected by the absence or presence of added glucose in the culture medium but was influenced by the concentration of serum in the suspending medium.

(D) The Effect of Prior Exposure of Macrophages to Cigarette Smoke on Antibacterial Activity: To assess the effect of pre-exposure to whole cigarette smoke on macrophage function, macrophage cultures were exposed to cigarette smoke for 1.5 hours and then challenged with bacteria. Under these circumstances of smoke-exposure, studies were also performed to determine if changes in the antibacterial activity of smoke-exposed macrophage were reversible under in vitro conditions of recovery, namely, incubation of smoke-exposed tissue culture preparations in smoke-free environment prior to bacterial challenge. For this purpose, macrophage cultures were exposed to whole cigarette smoke for 1.5 hrs. At this time, the tissue culture flasks were flushed out with sterile room air and the culture media was removed with a pipette. Fresh media was then added to the tissue culture flask and the macrophage cultures were then incubated for 1.5 hours in the absence of smoke prior to bacterial challenge. The data presented in Tables XV and XVI clearly demonstrates that prior exposure to 8 ml. of whole cigarette smoke caused an impairment of phagocytosis and bacterial killing that was not reversed by introducing an interim period of incubation in a smoke-free environment prior to bacterial challenge.

Conclusions: Exposure of alveolar macrophages to whole cigarette smoke under in vitro conditions of smoke-exposure caused an irreversible impairment of phagocytic and bactericidal powers of alveolar macrophages.

(E) The Effect of Multiple Washings of Smoke-Exposed Alveolar Macrophages on Cigarette Smoke Induced Changes in Antibacterial Activity: Studies of the reversibility of the adverse effect of cigarette smoke on macrophage function were performed under in vitro conditions of recovery. The macrophage cultures were first exposed to cigarette smoke for 1.5 hours. At this time, the tissue culture flasks were flushed out with sterile room air, the culture media removed and the adhering macrophages washed 3 additional times with Hanks' solution. Fresh Hanks' solution was then added to the tissue culture flasks and the macrophage cultures challenged with S. aureus for 1.5 hours in the absence of cigarette smoke.

The data presented in Table XVII clearly demonstrate that prior exposure to cigarette smoke caused an impairment of phagocytosis and bacterial killing that was partially, but not totally, reversed by multiple washing of smoke-exposed macrophage cultures. As shown in Table XVII, compared to appropriate controls, the percent bacteria killed by unwashed and washed smoke-exposed macrophages was decreased 29% and 19%, respectively.

Conclusions: Multiple washing of alveolar macrophages after smoke-exposure reduces but does not totally reverse the adverse effect of cigarette smoke on macrophage function.

(F) The Effect of Filtered Cigarette Smoke on the Antibacterial Activity of Alveolar Macrophages: Studies were performed with macrophage cultures exposed to whole cigarette smoke drawn through a glass fiber filter disc**. The filtered smoke was introduced immediately after bacterial challenge and maintained for 1.5 hours. As shown in Table XVIII, 8 ml. of filtered cigarette smoke caused less of an impairment of macrophage function than 8 ml. of whole smoke. In addition, 8 ml. of filtered cigarette smoke did not interfere with phagocytosis but impaired intracellular destruction of ingested bacteria. The data also indicates that 16 ml. of filtered smoke were required to produce the same interference of phagocytosis and bacterial destruction caused by exposure of macrophage cultures to 8 ml. of whole smoke.

Conclusions: Removal of the particulate phase of whole smoke reduced the toxic effect of cigarette smoke on the antibacterial activity of alveolar macrophages. The particulate and gas phases of whole smoke may have distinct effects on macrophage function.

IV. The Influence of Extended Exposure to Puffed Cigarette Smoke on Pulmonary Defense against Inhaled Staphylococcus aureus under In Vivo Conditions of Bacterial Challenge and Smoke-Exposure:

(A) The Effect of Cigarette Smoke on Lung Clearance: For this purpose experimental and control mice were first exposed to cigarette smoke and a secondary air flow of room air and then challenged with aerosols of Staphylococcus aureus for 30 minutes. Immediately after aerosol exposure groups of smoke-exposed and control mice were sacrificed to determine the number of bacteria deposited in their lungs. The remainder were killed 1 hour and 4 hours after bacterial challenge to ascertain the number of viable bacteria remaining in the lungs of smoke-exposed and control mice. These studies were performed immediately after daily exposure to cigarette smoke for 1 hour for 3, 5, 10 and 15 days. As shown in Table XIX, exposure to cigarette smoke for 15 days caused an impairment of 1 hour clearance; control animals cleared 48% of the bacteria and smoke-exposed 28%. In contrast, the 4 hour clearance rates of control and smoke-exposed were comparable; 84% and 82%, respectively. Comparable results were obtained in studies in which mice were exposed to cigarette smoke for 3, 5 and 10 days (Tables XX - XXII).

Conclusions: Exposure to Cigarette Smoke caused an impairment of bacterial clearance that was reversible within hours.

(B) The Effect of Cigarette Smoke on Basal Macrophage Yields: The effect of cigarette smoke on the numbers of alveolar macrophages harvested from the lungs of mice was studied by comparing the macrophage yields obtained from mice exposed to a secondary air flow (Control Group) and mice challenged daily for 1 hour with puffed cigarette smoke for 15 days. The data presented in Table XXIII demonstrates that smoke inhalation provoked a 2.6 fold increase in macrophage numbers and did not adversely effect cell viability (Table XXIV).

Conclusions: Smoke inhalation caused a marked increase in basal macrophage numbers and did not adversely effect cell viability.

(C) The Effect of Cigarette Smoke on the Mobilization of Alveolar Macrophages in Response to a Bacterial Challenge: These experiments were performed with control and experimental mice exposed to a secondary airflow and puffed cigarette smoke daily for 1 hour for 15 days. Basal macrophage yields were obtained from groups of control and smoke exposed mice and the remainder were exposed to an aerosol of S. aureus for 30 minutes. Immediately after aerosol exposure separate groups of control and smoke-exposed animals were sacrificed to determine bacterial deposition and mobilized macrophage

** Cambridge glass fiber filter, Phipps and Bud, Richmond, Va.

yields. As shown in Table XXV, the mobilized macrophage yields obtained from control and smoke exposed animals immediately after bacterial challenge were comparable.

Conclusions: Extended exposure to cigarette smoke did not mitigate the mobilization of alveolar macrophages in response to a bacterial challenge.

(D) The Effect of Cigarette Smoke on the Antibacterial** Activity of Alveolar Macrophages: In order to compare the antibacterial activity of smoke-exposed and control rabbits, experimental and control animals were exposed daily for 1.0 hour to cigarette smoke and a secondary air flow, respectively, for 15 days. Alveolar macrophage harvests from both categories of animals were treated as follows: washed five times with Hanks' solution, placed in tissue culture flasks containing Hanks' solution and 2.5% normal rabbit serum and challenged with S. aureus. As shown in Table XXVI, alveolar macrophages harvested from smoke-exposed macrophages phagocytized and destroyed staphylococci as effectively as macrophages from control animals.

Conclusions: The antibacterial activity of alveolar macrophages harvested from smoke-exposed animals and washed several times prior to bacterial challenge was unimpaired.



** % changes in antibacterial activity of smoke-exposed macrophages derived as follows: % phagocytosis by control minus % phagocytosis by smoke-exposed; % killed by controls minus % killed by smoke-exposed macrophages.

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9. Details of Experimental Design and Procedures: (Cited references on pages 2A-31 to 2A-33).

(I) Methods of Procedure:

A. Smoke-Exposure System: A smoke generating apparatus is used to deliver puffed cigarette smoke under controlled conditions. In the studies proposed herein, the apparatus will be adjusted to deliver a 35 ml puff of 2 seconds duration from a commercial brand of non-filtered cigarettes. The time interval between puffs from a single cigarette will be set at 58 seconds. The instrument is equipped with a rotating disc which can accommodate 30 cigarettes at one time, so that it is possible to maintain a continuous stream of puffed smoke generated at a rate of 1 L/min. The initial smoke delivered by the cigarettes is diluted and transported across an animal exposure chamber by a secondary air flow. Control animals are placed in a plexiglass chamber and exposed to a secondary air flow. These are the same conditions of smoke exposure used to assess the influence of acute and extended exposure to puffed cigarette smoke on bacterial clearance and alveolar macrophage function in studies reported under Supporting Data, pages2A-7 to 2A-8. In studies with filtered cigarette smoke, the smoke generating apparatus will be modified to include the insertion of a glass fiber filter disc*. A gas partitioner will be used to monitor the total hydrocarbon content in the smoke-exposure chamber. The tar concentration of smoke will be determined by established methods. Air samples from the smoke exposure chamber will be collected on filter paper, weighed, extracted in alcohol and subjected to spectrophotometric assay.

B. Conditions of Animal Smoke-Exposure: Animals will be exposed daily for 1.0 hour /for 3 weeks to whole cigarette smoke or cigarette smoke passed through a glass fiber disc. The maximum number of total days of smoke-exposure planned over the entire 3 week study period is 15 days. However, total days or daily length of smoke-exposure will be reduced if toxicity, animal death or bacterial contamination of broncho-pulmonary tissue are noted. The daily length of exposure corresponds to the smoke-exposure periods previously used to study the effect of acute exposure to cigarette smoke tracheal clearance, lung clearance and alveolar macrophage activity. Under these conditions, it will be possible to determine the effect of whole and filtered cigarette smoke on pulmonary defense and to correlate these observations with the data obtained to date under conditions of acute and extended exposure to cigarette smoke (see Supporting Data, pages 2A-3 to 2A-5). The proposed protocol is similar to that used by LaBelle et al (1) to study the effects of acute and extended exposure to cigarette smoke on pulmonary clearance of radioactive test particles.

C. Bacterial Aerosol Exposure Unit: Since bacterial clearance studies comprise a major part of the proposed research project, a review of the method of aerosol formation, exposure and quantitative aspects of bacterial clearance is indicated. Aerosols are generated from a buffered suspension (pH 7.3) of staphylococci contained in 3 glass nebulizers. The initial spray from the nebulizers is directed into mixing chambers through which a secondary air flow of 100 cubic feet/min is drawn. The large volume of secondary air serves to mix, dilute and dry the initial bacterial aerosol; direct it past an interposed baffle for removal of large droplets; and then carry it through a large plexiglass exposure chamber that can accommodate up to 200 mice. The particle size distribution of the bacterial aerosol is monitored with an Andersen Sampler (2). White male Swiss Webster mice are divided into groups of 5 to 10 animals and exposed to Staphylococcal aerosols for 30 min. Immediately after exposure (0 time) one group of animals is sacrificed to determine the numbers of viable bacteria deposited in the trachea and lungs, respectively. The trachea and lungs are removed as separate blocks and individually ground in glass homogenizers. The remaining groups

of challenged mice are killed and processed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after aerosol exposure. Bacterial counts are obtained from nutrient agar pour plates of lung and tracheal tissue homogenates. By this method paired studies of lung and tracheal clearance of bacterial deposits are possible. By subtracting the mean number of culturable bacteria retained in the lung at each interval during the post aerosol exposure period from the number originally deposited (0 time), the mean number of staphylococci cleared by the lung may be derived. From these data lung clearance rates are calculated by expressing the number retained in the lung as a percentage of the deposition number (0 time) and subtracting this value from 100%. Similarly, from the numbers of bacteria present in the trachea at 0 time and at the same intervals after aerosol exposure, the numbers and percent bacteria cleared by the trachea may be obtained.

D. Harvesting of Alveolar Macrophages: In order to correlate macrophage activity with bacterial clearance, a method has been developed in this laboratory for harvesting alveolar macrophages from the murine lung (3,4). Mice are sacrificed and the trachea and lungs exposed. The intact lung is washed by 5 successive 1.0 ml washes with Hanks' solution and the harvested cells recovered by centrifugation. By this method, it is possible to obtain 1.0 to 2.0 x $10^5$ macrophages from each mouse's lung with 90% viability. Total cell counts are performed in a bright line hemocytometer and differential counts are made on Wright stained smears. Cell viability is assessed by the capacity of alveolar macrophages to reject the stain Eosin I. The number of alveolar macrophages that are available in harvest by this technique under basal conditions is referred to as a basal yield. Therefore, mobilization is taken to represent the increase in macrophage numbers over basal levels harvestable from the lungs after a bacterial challenge. The mobilization of alveolar macrophages may be quantitated by this technique. Studies completed in our laboratory to date indicate that macrophage yields are increased 1.5 times basal levels after exposure to aerosols of a phosphate buffer or dead Staphylococcus aureus (4), and 2 to 3 times basal levels in response to aerosols of viable S. aureus (3,4). During the post-aerosol exposure period there is an initial drop in macrophage numbers but elevated levels are restored in 30 min. and this increase is maintained for 4 hours (4). This sustained response is blocked by alcohol, but not by acute exposure to cigarette smoke (4,3) (See Supporting Data, pages2A-3to2A-5).

Alveolar macrophages are harvested from the lungs of albino rabbits weighing 1.0 to 2.0 kilograms by the general method of Myrvik et al (5). The animals are killed by injecting air in the marginal ear vein. This method of sacrificing animals is used to avoid any depressant effects that anesthetics may have on alveolar macrophage function. The trachea and lungs are exposed, and macrophages harvested by washing out the intact lungs with 17 ml. of Hanks' solution. The harvested cellular contents of the lungs are recovered by centrifugation at 2000 rpm for 20 minutes. Total cell counts are performed in a bright line hemocytometer, and differential counts are made on Wright stain smears. By this method, 95% of the macrophages harvested are viable as determined by the Eosin I technique (6).

E. Acellular Fractions of Lung Harvests: Recent studies suggest that secreted fluids present in the bronchopulmonary tree may independently or in concert with alveolar macrophages play a significant role in pulmonary defense against inhaled bacteria. In this regard, alveolar living material (ALM) has been reported to enhance the bactericidal activity of rat alveolar macrophages (7). Secretory IgA is present in the tracheobronchial washing of normal patients (8) and has the capacity to kill Escherichia coli (9) and inhibit the adherence of certain strains of Streptococci to epithelial tissue (10). For these reasons, the acellular fractions of lung harvests (ACF) will be collected, concentrated, Secretory IgA levels monitored and ALM isolated.

In addition, the antibacterial and phagocytosis properties of concentrated acellular fraction and AIM against S. aureus will be assessed in the phagocytosis system detailed on page 2A-9. These studies will be performed with acellular fractions of lung harvests obtained from control and smoke-exposed animals.

The method of LaForce et al (7) will be used to isolate alveolar lining material from the lungs of rabbits. Rabbits are sacrificed by injecting air in the marginal ear vein and the trachea and lungs are exposed. The trachea is canulated with a sterile polyethylene tube, and (0 ml of sterile heparinized saline (10 units per ml) are introduced in the lung and recovered by aspiration. The recovered bronchoalveolar saline lavage fluid is centrifuged at 800 g for 8 minutes; the supernatant decanted and saved. The cell free supernatant is centrifuged at 40,000 g at 4°C for 20 minutes. The recovered precipitated pellet represents the alveolar lining material or surfactant fraction (11).

In order to study, the antibacterial and phagocytosis promoting characteristics of the entire acellular fraction of lung harvests, bronchoalveolar saline lavage fluid recovered from rabbit lungs will be initially concentrated by ultrafiltration (Diaflow Membranes, Amicon Corp) and fractionated by gel column chromatography.

The presence of Secretory IgA (SIgA) in bronchoalveolar washings will be monitored by double immunodiffusion (12) against anti-SIgA and anti-secretory piece sera. SIgA levels will be quantitated by single radial immunodiffusion (13) utilizing anti-SIgA serum impregnated in the agar gel and SIgA as the antigen standard. The SIgA antigen standard will be prepared from clarified colostrum (14). The latter procedure includes: (a) separation by gel chromatography and further purification by anion-exchange chromatography using a stepwise elution gradient of phosphate buffers of varying ionic strengths. The purity of SIgA will be assessed by disc electrophoresis (15).

(II) Details of Experimental Design of Proposed Research Plan: In keeping with the intended purpose of the proposed research, a series of studies are planned to evaluate the effect of extended exposure to whole and filtered cigarette smoke on tracheal and lung clearance of bacterial deposits. All studies will be performed under in vivo conditions of smoke-exposure.

A. Paired Observations of Tracheal and Lung Clearance: For this purpose, experimental and control mice will be first exposed to cigarette smoke and a secondary air flow, respectively, and then challenged with aerosols of S. aureus for 30 minutes. Immediately following aerosol exposure (0 time) some of the smoke-exposed and control animals will be sacrificed to determine the number of bacteria initially deposited in their trachea and lungs. The remainder will be killed 15 min, 30 min, 1 hr, 2 hrs and 4 hrs after bacterial challenge to ascertain the number of viable bacteria remaining in the trachea and lungs of smoke-exposed and control mice. From this data, it is possible to calculate the rate at which bacteria are cleared from the trachea and lungs. These studies will be performed in mice exposed daily for 1.0 hours to cigarette smoke over a 3 week period. Tracheal and lung clearance rates will be measured immediately following and 24 to 48 hrs after daily exposure to cigarette smoke for 15 days. The following specific information is sought from these studies: (a) the effect of extended exposure to whole and filtered cigarette smoke on tracheal and lung clearance of bacterial deposits and (b) the reversibility of any adverse change in clearance rates attributable to smoke-exposure. Since this aspect of the proposed research will be completed by the end of the current grant support, a limited number of clearance studies will be performed to audit the conditions of smoke exposure

established in the alveolar macrophage studies detailed in the current grant proposal and to correlate the data obtained from these observations with the clearance and macrophage data compiled to date (See Supporting Data, pages 2A-3 to2A-5).

B. Alveolar Macrophage Activity: These studies will be performed under the same conditions of smoke-exposure used to assess the effect of extended exposure to whole and filtered cigarette smoke on tracheal and lung clearance. In this regard, mice and/or rabbits will be exposed daily for 1.0 hrs to cigarette smoke over a 3 week period and alveolar macrophage activity will be assessed immediately following and 24-48 hrs after daily exposure to smoke for 15 days.

(1) Phagocytosis and Intracellular Killing Activity: Studies will be performed to determine the effect of extended exposure to whole and filtered cigarette smoke on the antibacterial activity of alveolar macrophages in the intact and functioning lung. For this purpose, large numbers of experimental and control mice will be first exposed to cigarette smoke and a secondary air flow, respectively, and then challenged with staphylococcal aerosols for 30 minutes. Immediately after bacterial challenge (0 time) and at hourly intervals during a 4 hour post-aerosol exposure period, alveolar macrophage harvests obtained from smoke-exposed and control animals are processed as follows: bacterial counts are obtained from an aliquot of the total lung washout and the remainder is separated by differential centrifugation at 1500 rpm for 15 min into a supernatant fraction containing free bacteria and a cellular fraction ladened with alveolar macrophages and phagocytized bacteria. The number of viable bacteria present in each fraction is determined by a standard pour plate technique. The decrease in the number of viable bacteria present in the total lung washout, as a function of time, is used as an index of the clearance rate of the lung sample obtained by the lavage technique. Similarly, a decrease in the viable counts of the supernatant and cellular fractions as a function of time, are used as a measure of the quantitative phagocytic and intracellular killing activity, respectively, of alveolar macrophages. These studies will be performed under the same conditions of smoke-exposure as used to assess the effect of extended exposure to whole and filtered cigarette smoke on bacterial clearance. In this regard, antibacterial activity will be assessed immediately following and 24 hours after daily exposure to cigarette smoke for 15 days. Under these conditions it will be possible to compare the effect of whole and filtered cigarette smoke on the antibacterial activity of macrophages under in vivo conditions of bacterial challenge and to assess the relative influence of the particulate and gas phases of smoke on this important parameter of macrophage function. The protocol will also permit an assessment of the reversibility of any impairment in phagocytic and bactericidal powers that may occur as a result of exposure to cigarette smoke.

In a separate series of studies, alveolar macrophages harvested from smoke-exposed and control mice and rabbits will be challenged with S. aureus in an in vitro phagocytosis system. The methods, procedures and details of the in vitro phagocytosis are presented in detail in the Methods and Materials Section of Supporting Data, page 2A-9. Fixed numbers of staphylococci are added to known numbers of macrophages adhering to the flat surface of a tissue culture flask containing Hanks' solution (0.1% glucose). Immediately after bacterial challenge and at various intervals thereafter, the extracellular fraction containing free or unphagocytized bacteria is isolated by removing the supernatant fluid from macrophage cultures with a pipette. To recover the macrophage fraction, the tissue culture flask is vigorously shaken after adding glass beads and distilled water. Bacterial counts are obtained from each fraction by a standard pour plate technique. By this method, it is possible to determine the percent bacteria phagocytized, percent intracellular survival of phagocytized bacteria and

percent bacteria killed by macrophages. The purpose of these studies is to obtain data that will permit a better understanding of the antibacterial activity of alveolar macrophages in the intact and functioning lung under normal conditions and during the inhalation of cigarette smoke. For this reason studies are planned to evaluate the influence of increased macrophage numbers, opsonization of bacteria, pre-treatment of bacteria with alveolar lung material, pre-treatment of bacteria with the total acellular fraction of lung harvests on the phagocytic and bactericidal powers of alveolar macrophages. In separate studies alveolar macrophages will be challenged with S. aureus and incubated at 37°C in tissue culture flasks containing Hanks' solution supplemented with the following: (a) normal serum with and without specific immune serum added, (b) alveolar lining material (ALM) alone, ALM plus normal and immune serum, ALM plus immune serum and ALM plus normal serum and (c) concentrated acellular fraction (CAF) alone, CAF plus normal and immune serum, CAF plus immune serum and CAF plus normal serum. This information is needed for proper analysis of the events observed under in vivo condition of an airborne bacterial challenge and is in keeping with recent observations that suggest local immune systems and pulmonary secretion play a significant role in pulmonary defense (7,8) and, as such, may act as mediators of alveolar macrophage function. In addition, the above information will be used to establish in vitro culture conditions necessary for meaningful studies of macrophage mobilization and the metabolic activity of alveolar macrophages outlined below; namely, in vitro culture conditions that best reflect the in vivo circumstances in the live and intact lung.

(2) In Vitro Studies of Cell Adhesiveness and the Motility and Migratory Response of Basal and Mobilized Alveolar Macrophages to Chemotactic Substances: For this purpose, a method of studying leukocyte motility reported by Carruthers (16) will be adapted to alveolar macrophages. This method is based on the ability of motile cells to move through the pores of a membrane filter. In this regard, two O-ring joints separated by a millipore filter are clamped together and sealed which results in the formation of 2 distinct chambers. Fixed numbers of alveolar macrophages (1-3 x $10^7$) suspended in Hanks' solution (3 ml.) containing glucose will be introduced into chamber number one which is then sealed with a paraffin plug. The second chamber is filled with 5 mg. of insoluble potato starch and plugged. After an initial period of incubation at 37°C to permit monolayer formation, the chambers are inverted so that the test cells are now on the bottom side of the filter, and the chemotactic substance, if present, is on the top side of the filter. The chamber is then placed in an incubator at 37°C for 4 hours. At the end of various hourly intervals, the filter is removed, stained with hematoxylin and subjected to microscopic study. In this way, the number of cells on both sides of the filter are enumerated and used as an index of the migratory response elicited by stimulatory agents. Separate studies are planned to evaluate the changes in cell adhesiveness that occur in phagocytizing alveolar macrophages. The method of studying the adhesive properties of blood leukocytes reported by Allison and Lancaster (17) will be adapted to alveolar macrophages. Test tube cultures of fixed numbers of lung phagocytes suspended in Hanks' solution will be challenged with known numbers of bacteria. Under conditions favoring maximum phagocytosis, microscopic methods will be used to determine the formation of cell aggregates that clump together by cultures of phagocytizing macrophages. Changes in cell adhesiveness will be evaluated under the same experimental conditions described above to assess motility.

An understanding of this aspect of alveolar macrophage activity is desirable because motility and changes in cell adherence may be critical steps in the mobilization and migration of lung phagocytes to pulmonary sites, as well as their subsequent antibacterial activity during the normal situation and in response to the

inhalation of viable bacteria and cigarette smoke. The data obtained from these studies will be correlated with the results of studies completed in this laboratory concerning the mobilization of alveolar macrophages in response to the inhalation of viable bacteria in the normal situation and during the inhalation of cigarette smoke. (See Supporting Data, Results pages 2A-10 and 2A-15).

(3) Metabolic studies: The present grant will include studies of the energy metabolism, lipid compositions, hydrolytic enzyme activity and peroxidative metabolism of basal and mobilized alveolar macrophages harvested from control and smoke-exposed animals. This information is desirable, since energy output and cell metabolism represent important potential links in the successful mobilization and subsequent antibacterial activity of alveolar macrophages. Indeed, alterations in hydrolytic enzyme activity and peroxidative metabolism may interfere with the primary immunological function of macrophages in pulmonary defense, namely, the destruction of inhaled microorganisms. In addition, interest in the lipid fraction of phagocytic cells resides in the fact that lipids represent a source of high energy metabolism and are intimately involved in membrane phenomena associated with phagocytosis. The metabolic studies will be performed under the same culture conditions (suspending medium) used to assess the influence of cigarette smoke on the phagocytic and bactericidal capacity of alveolar macrophages.

Respiration studies: Experiments are planned which will permit determinations of the oxygen consumption and lactic acid content of alveolar macrophages incubated in basal medium and media supplemented with glucose plus serum with and without the presence of particles that induce phagocytosis. In separate studies serum will be supplemented with and/or replaced by alveolar lining material and the concentrated acellular fraction of lung harvests as outlined in the phagocytosis studies detailed on page2A-24. These studies will be performed with the following categories of macrophages: (a) basal macrophages harvested from control and smoke-exposed animals and (b) mobilized macrophages harvested from control and smoke-exposed animals immediately after exposure to aerosols of a phosphate buffer for 30 minutes.

Measurements of oxygen uptake will be determined in a Gilson respirometer using flasks with a 15 ml capacity containing monolayers of alveolar macrophages in a total liquid volume of 3.2 ml (18). The $CO_2$ will be absorbed by 0.2 ml of 20% KOH in the center well. In accordance with the protocol of each study, glucose (5.0 to 10 mm) and polystrene spheres at a concentration of 2.0 to 2.5 mg/ml will be introduced via the side arm. After completion of the oxygen consumption measurements, the cells will be harvested and their lactic acid content determined by the method of Barker et al(19). For this purpose, the recovered macrophages will be washed in saline and cell extracts are to be prepared as described by Myrvik et al (20).

Glucose metabolism studies: Experiments with specifically labelled glucose as substrate will be performed to evaluate the effect of cigarette smoke on the metabolism of glucose by alveolar macrophages. These studies will be done with the same categories of basal and mobilized macrophages used in the respiration studies.

The radioactive measurements will be done as described by Myrvik et al (20). Alveolar macrophages are harvested and placed in Erlenmeyer flasks containing medium 199 without serum or glucose. In separate experiments BCG and heat killed staphylococci will be added to all flasks except the control flasks. After incubation at 37°C in a shaker bath for 1, 2, 4, 6 and 18 hours, glucose 1-$C^{14}$ or glucose 6-$C^{14}$ is added to the flasks, and reincubated for 1 hour. The reaction is stopped with sulfuric acid and counts obtained with a liquid scintilation counter.

ATPase Activity: Experiments are planned to compare the ATPase activity of basal and mobilized alveolar macrophages harvested from control and smoke-exposed animals. ATPase activity will be determined from the liberation of inorganic phosphate (Pi) upon incubation of alveolar macrophages with adenosine triphosphate (ATP) as outlined by Wahler et al (a1). The assay medium will contain 100 mM sucrose, 30 mM glyclycine, 30 mM imidazole, 5 mM $MgCl_2$, 2 mM ATP, protein equivalent of cells 50 to 100 ug and, as indicated, 50mM NaCl plus 5 mM KCL (pH 7.5). In studies performed in the absence of $Na^+$ and $K^+$, the medium will contain 200 mM sucrose. Reactions will be carried out in a 2nd volume for 20 minutes at 30°C and activity will be expressed as micromoles of Pi liberated per milligram of protein per hour.

Catalase Activity, peroxidase activity and hydrogen peroxide production: Recent studies (22) have presented evidence for the presence of a catalase-dependent peroxidative metabolism. Peroxidative metabolism represents a biochemical pathway capable of increasing glucose metabolism and hydrogen peroxide formation. In this regard, published reports clearly demonstrate that phagocytosis by normal alveolar macrophages is accompanied by increased glucose metabolism (35) and intracellular recovery of hydrogen peroxide (22). The potential role of catalase controlled concentrations of hydrogen peroxide as an intracellular bactericidal agent has been recognized. For these reasons studies are proposed to compare the catalase activity, peroxidase activity and hydrogen peroxide production in macrophages harvested from control and smoke-exposed rabbits. These studies will be performed with basal macrophages, and macrophages mobilized in response to the inhalation of heat-killed staphylococci harvested from both control and smoke-exposed animals.

Catalase activity will be determined by the method of Feinstein (23) using 0.1N sodium perborate as substrate. Perborate utilization in 5 min is measured by titration with a 0.1N solution of potassium permanganate after the reaction is stopped with 1N sulfuric acid. Catalase activity will be determined after incubation in Krebs Ringer Phosphate Solution containing 15% homologous serum and 5.5 mM glucose at pH 7.4. Measurements will be performed on macrophage preparation after disruption by either homogenization or repeated freeze-thawing using acetone-dry ice. Both total extract and supernatant obtained by centrifugation at 8000 rpm for 10 min will be assayed. Activity will be expressed as milliequivalents of perborate utilized in 5 min, 1 U representing the utilization of 1 mEq of perborate. Peroxidase activity will be assessed by a modification of the guaiacol method of chance and Maehly (24). Whole extracts of freeze-thawed cells will be employed. The assay medium contains 0.1 M phosphate buffer at pH 7.4, 0.5 ml of 100 mM guaiacol, 0.2 ml of extract sample, and 0.02 ml of ice-cold 10 mM $H_2O_2$. Absorbancy changes due to tetraguaiacol formation will be measured at 750 nm in a spectrophotometer and the time required to produce an 0.05 U increase in absorbancy recorded. Results will be expressed in reciprocal seconds per $10^9$ cells.

Hydrogen peroxide will be determined spectrophotometrically on dialysates of AM as described by Paul and Shara (25). The nonfluorescent dye, diacetyl-2,7-dichlorofluorescin (LDADCF), was synthesized by the method of Brandt and Keston (26) and the fluorescence of the oxidized product of alkali-activated LDADCF was measured with an Aminco-Bowman spectrofluorimeter. The excitation wave length was 340 nm and the emission wave length 525 nm.

Hydrolytic enzyme studies: The present grant proposal will include a study of the enzymatic activity and intracellular distribution of a specific group of hydrolytic enzymes in basal and mobilized macrophages harvested from control and smoke-exposed animals. The enzymes acid phosphatase, lysozyme, lipase, beta-glucuronidase and cathepsin are of immediate interest because their activity is increased

in the ECG induced alveolar macrophage (17, 28-29). To accomplish these aims, the enzymatic activity detectable in the cell free supernatant fraction and alveolar macrophage fraction of lung harvests will be assayed. Interest in both fractions of lung harvests resides in the fact that bronchial mucus contains several poorly defined substances (30) including lysozyme (31) that exert nonspecific bacteriostatic and bactericidal activity against gram positive and gram negative bacteria as well as the potential phagocytosis promoting factor present in pulmonary secretions (7,8). In addition, alveolar macrophages by virtue of their high hydrolytic enzyme content, presence in large numbers and rapid turnover rate may contribute to the enzymatic activity found in mucus secretions.

A comparison will be made of the enzymatic activity and intracellular distribution of the above hydrolases in the following categories of macrophages: (a) basal macrophages harvested from control and smoke-exposed animals and (b) mobilized macrophages harvested from control and smoke-exposed animals immediately after exposure to aerosols of viable or heat-killed staphylococci. For this purpose, smoke-exposed and control animals will be exposed to the bacterial aerosols for 30 minutes. Enzyme determinations will be made with the lung harvests obtained from both groups of animals immediately after bacterial challenge and at hourly intervals over a 4 hour post-aerosol exposure period. The choice of laboratory animals (mice or rabbits) to be used in all enzyme studies will be governed by the minimal yields necessary for adequate enzyme assay. The lung washing obtained from animals by the lavage technique will be separated into a cell-free supernatant fraction and cellular fraction containing alveolar macrophages by centrifugation at 4°C for 15 min at 10,000 rpm (31). The supernatant will be frozen at -60°C and stored until assay. The harvested alveolar macrophages will be washed twice in phosphate buffered saline (pH 7.2); quantitated by hemocytometer count; and viability assessed. Saline extracts of the washed cells are to be prepared for enzyme activity studies as outlined by Myrvik et al (20) cell disruption by alternate freezing and thawing for 5 consecutive cycles and removal of cellular debris by centrifugation at 2,500 rpm for 10 min at 4°C. The completeness of cell disruption will be audited by phase optics. In order to evaluate the contribution made by alveolar macrophages to the enzymatic activity detectable in the supernatant fraction, in vitro studies of the rate of release of the enzymes in question by pulmonary macrophages are planned. As outlined by Holzman et al (31) large numbers of alveolar macrophages will be suspended in tissue culture medium and samples of the cell population assayed for specific enzymatic activity after incubation at 37°C for various periods of time. In studies of the intracellular distribution of the hydrolases, alveolar macrophages will be suspended in a 0.25M sucrose solution, ruptured by homogenization and sedimented by centrifugation into four fractions (27). The nuclear fraction (N) will be sedimented by centrifugation at 250 X G; the heavy granule fraction (HG) at 5000 X G for 15 min; and the light granular fraction (LG) and supernatant fraction (S) by centrifugation at 25,000 X G. Each fraction will be submitted to 5 cycles of freezing and thawing and clarified by centrifugation at 2500 X G for 20 min and subjected to enzyme assay.

For the purpose of enzyme analysis, lysozyme will be quantitated by using suspensions of Micrococcus leisodykticus as substrate. Tests will be standardized with known amounts of crystalline egg white lysozyme and results expressed as egg white lysozyme equivalents. Acid phosphatase will be measured by the procedure of Hofstee (32) using O-carboxyphenyl phosphate as substrate. An increase in absorbance of 0.001 optical density units/min under standard conditions will be taken to represent a unit of activity. Beta-glucuronidase will be assayed by the procedure of Fishman et al (33) using phenolphthalein mon-Betaglucuronide as substrate with reactions carried out in 0.1M acetate buffer at pH 4.5 and 38°C. One unit of glucuronidase is

the activity resulting in the liberation of 1 ug/hr phenolphtalein. Lipase activity will be determined as outlined by Cohn et al (28) in which naphthol laurate serves as substrate and the increase in activity caused by sodium taurocholate is taken to represent minimal lipase activity. The results are to be expressed as micromoles of naphthol liberated per hour. Cathepsin will be assayed employing a 2% solution of denatured hemoglobin as a substrate, as described by Anson (34) and protein digestion estimated with a spectrophotometer by absorption at 280 nm (27). A unit of activity being defined as an increase in optical density produced by 0.001 meq of tyrosine.

Lipid composition of basal and mobilized alveolar macrophages: The specific information sought from these studies in the qualitative and quantitative changes in the major lipid classes and fatty acid composition of alveolar macrophages associated with mobilization and phagocytosis in the normal activation and during the inhalation of cigarette smoke. To accomplish these aims a series of in vivo and in vitro studies are planned.

A comparison will be made of the lipid and fatty acid components extractable from alveolar macrophages harvested from unchallenged animals and animals exposed to aerosols of S. aureus. Alveolar macrophages will be harvested from the challenged animals immediately after aerosol exposure and at hourly intervals thereafter over a 6 hr. period. Since the presence of bacteria within alveolar macrophages may influence the outcome of these studies, appropriate controls will be used; namely, cells provoked by inert particles. In addition, S. aureus, in numbers equivalent to those deposited in the lungs of the test animals, will be carried through the same lipid extraction and analysis. These studies will be repeated with animals which have been exposed to cigarette smoke and cigarette smoke plus bacterial aerosols. A series of studies will be undertaken to determine the effects of metabolic inhibitors on the lipid composition of basal and phagocytizing alveolar macrophages. The specific information sought from these studies is as follows: (a) insight into the energy-yielding metabolic pathways utilized by alveolar macrophages during phagocytosis and (b) the correlations that exist between the effects of metabolic inhibitors on phagocytosis and the lipid composition of these cells. The rationale behind this approach resides in the observation that alveolar macrophages undergo a minimal increment in metabolic levels ($O_2$ uptake and carbohydrate metabolism) during phagocytosis It is therefore proposed that an end result of metabolic activity, namely lipid composition, be studied. The specific metabolic inhibitors to be used are those which block glycolysis (monoiodoacetate, sodium fluoride) Kreb's cycle activity (sodium malonate, sodium fluoroacetate) and respiratory inhibitors cyanide, (dinitrophenol and anaerobiosis) and others as indicated by the initial results observed. The concentrations of inhibitors to be used will be determined empirically as those concentrations which interfere with phagocytosis and alter lipid composition. However, the concentrations used by Oren et al (18), Karnovsky et al(35) and Ouchi et al(36) in evaluating the effect of these metabolic inhibitors in the metabolic levels of phagocytizing cells will be referred to. The protocol to be followed in the in vitro studies consist of incubating known numbers of alveolar macrophages in a balanced salt solution containing glucose, in tissue flasks at 37°C until monolayer formation occurs. At this time, polystyrene spheres (2.0 to 2.5 mg/ml, final concentration) and specific concentrations of metabolic inhibitors will be added to the monolayers. The mixture will be further incubated for 1 hour and harvested. An aliquot will be used for microscopic evaluation of phagocytosis and the remainder will be subjected to lipid analysis. The choice of laboratory animals to be used in all lipid studies will be governed by the minimal yields necessary for adequate lipid analysis. Total lipid extraction will be carried out by the method of Folch et al (37) which consist

of multiple additions of chloroform methanol mixtures to washed cells incubated at 55°C for 4 hrs. The separation of lipid classes will be performed by the techniques of Fillerup and Mead (38) which consists of liquid-solid chromatography on silicic acid. Separation of the various lipid classes (sterol esters, tri-glycerides, free sterols, free fatty acids and phospholipids) is achieved by a step-wise application of eluants of varying polarity. Thin layer chromatography will be used to assess the purity of each fraction eluted by column chromatography and to identify the different lipid classes present in total lipid extracts. The technique to be used is that of Malins and Mangold (39) in which thin layer plates coated with silica gel G are spotted with lipid material and developed in polar and non-polar systems. The resolved lipid spots are then visualized with an aqueous solution of rhodemine G or iodine vapors. The fatty acid composition of alveolar macrophages will be studied by converting the fatty acids collected by column chromatography and those present in total lipid extracts to their respective methyl esters and employing gas liquid chromatography.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Microbiology Research Laboratories are located as a complex in the Triboro Hospital at Queens Hospital Center, Jamaica, New York. They include: (a) an aerosol exposure laboratory, (b) a smoke-exposure laboratory, (c) individual laboratories for macrophage studies and microbiology, (d) 2 rooms for storage space and refrigerators. Animal quarters are provided in another area of the hospital. The entire laboratory area occupies approximately 700 square feet.

The equipment in this area includes a complete bacterial aerosol generating and exposure system with mixing chambers and decontaminating units, and an Anderson apparatus for measuring particle size of bacterial aerosols: A cigarette smoke generating apparatus and exposure chamber and a sequential sampler and gas liquid chromatographer unit for determining concentration of the particulate and gas phase of cigarette smoke. Other major instrumentation present include the following: (a) standard microscope, (b) 1 infusion pump, (c) a refrigerated centrifuge (d) 2 large refrigerators, (e) sonic dismembrator, (f) 2 water bath and shaker, (g) 1 freezer, (h) incubator and 1 environmental chamber, (i) 4 vacuum pumps, (j) pH meter, (k) Peckman DU2 recording spectrophotometer, (l) spectronic 20 spectrophotometer, (m) a Gilson respirometer and (n) a lyophilizer unit. There is also equipment for qualitative and quantitative bacteriology studies, tissue homogenization, administration of gas mixtures and animal surgery. A Revco deep freezer (-75°C) is also available. In addition, high performance scintillation counters capable of isotope work are present in the hospital and available for research use.

11. Additional facilities required: None



12. Biographical sketches of investigator(s) and other professional personnel (append): See attached Curriculum Vitae.
See pages 6 to 9.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
See pages 10 to 11.

14. First year budget:

| A. Salaries (give names or state "to be recruited") | hrs/wk % time | Amount |
|---|---|---|
| Professional (give % time of investigator(s) even if no salary requested) | | |
| Joseph J. Guarneri, Ph.D. Principal Investigator | 20 hrs./60 hrs. | None |
| Marcelino Sierra, Ph.D. | 7 hrs. | None |
| Research Associate, Ph.D. (to be recruited) | 100% | 15,000 |
| | Fringe Benefits | 2,550 |
| Technical | | |
| Amar Sarwal, M. S. | 100% | 11,288 |
| | | 1,919 |
| Research Lab Aide | 25% | 2,200 |
| | | 374 |
| | Sub-Total for A | 33,331 |

B. Consumable supplies (by major categories)

See page 4A for detailed list

| | |
|---|---|
| Sub-Total for B | 6,150 |

C. Other expenses (itemize)

| | |
|---|---|
| Travel | 700 |
| Publications | 150 |
| Sub-Total for C | 850 |
| Running Total of A + B + C | 40,331 |

D. Permanent equipment (itemize) See page 4A for justification

1. Sorval RC 2-B Automatic Superspeed Refrigerated centrifuge including rotors and heads ($4000)
2. Gel Electrophoresis Cell (Bio Rad) with Power Source ($555).
3. Fraction Collector - Isco Model 1200 pup ($600)
4. LKB Peristaltic Pump ($300)

| | |
|---|---|
| Sub-Total for D | 5,455 |
| E | 6,019 |
| Total request | 51,835 |

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Indirect Costs | Total |
|---|---|---|---|---|---|---|
| Year 2 | $35,831* | 6,150 | 850 | 1,000 | 6,425 | 50,256 |
| Year 3 | 38,518* | 6,150 | 850 | - | 6,828 | 52,346 |

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B. Consumable Supplies

| | | |
|---|---|---|
| 1. | Cigarettes for smoke studies | $1,750 |
| 2. | Mice Caesarian delivered | 1,000 |
| 3. | Rabbits | 1,200 |
| 4. | Immunodiffusion Plates, Anti SIgA and Anti Secretory Piece Sera, Radial Immunodiffusion kit and templates | 200 |
| 5. | Chromatography columns with 4 way valve, accessories, and Reagents for Chromatography, Sephadex G 200, Aerylamide kit, DEAE | 353 |
| 6. | Reagents and curvettes for enzyme studies and flasks and accessories for respiration studies | 500 |
| 7. | Tissue culture glassware, tissue culture media and bacteriology media | 647 |
| 8. | Isotopes uniformly labelled glucose - 1-$C^{14}$ and glucose 6-$C^{14}$ | 300 |
| 9. | Petri dishes and plastic disposables | 200 |
| | Total | $6,150 |

D. Permanent Equipment (Justification)

1. The Sorval RC 2-B Superspeed Refrigerated Centrifuge is needed for the alveolar lining material studies (ALM) proposed on pages 2A-22 and 2A-25 of grant request. The instrument and its rotors permit centrifugation up to 49,500 g with controlled temperature. The latter permits g forces and controlled conditions necessary for the isolation of ALM without the loss of biological activity.

2. Gel Electrophoresis Cell with a power source is needed for the purification and characterization of Secretory IgA and other proteins of immunological interest in the acellular fraction of lung harvests. See pages 2A-23 of the grant proposal for specific studies in which Gel Electrophoresis will be used.

3 & 4. Fraction collector and LKB Perisaltic pinup are needed for the purification and isolation of proteins in the acellular fraction of lung harvests and lipid studies (page 2A-23 and page 2A-30).

16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| The Influence of Extended Exposure to Cigarette Smoke on Pulmonary Resistance to Infection as Related to Alveolar Macrophages and Mucociliary Function. | The Council for Tobacco Research, U.S.A. Grant Nos. 547C, 547CR-1 and 547 CR-2. | $63,152 | 7/1/71 to 6/30/74 |

PENDING OR PLANNED

| Title of Project | Source (give grant numbers) | Amount | Inclusive Dates |
|---|---|---|---|
| Important Determinants to Pulmonary Resistance | Long Island Jewish-Hillside Medical Center (approved) | $36,340 | 7/1/74 - 6/30/76 |

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Joseph J. Guarneri, Ph.D.

Signature [signature] Date 1/14/74

Telephone 212-990-2335 -
Area Code Number Extension

Checks payable to

Long Island Jewish-Hillside Medical Center

Mailing address for checks

Mrs. Eva Meyer, Grant Manager
Long Island Jewish-Hillside Medical Center
New Hyde Park, New York 11040

Responsible officer of institution

Typed Name Harold Light

Title Deputy Director

Signature _____ Date _____

Telephone 212 343-6700 2723
Area Code Number Extension

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